



1

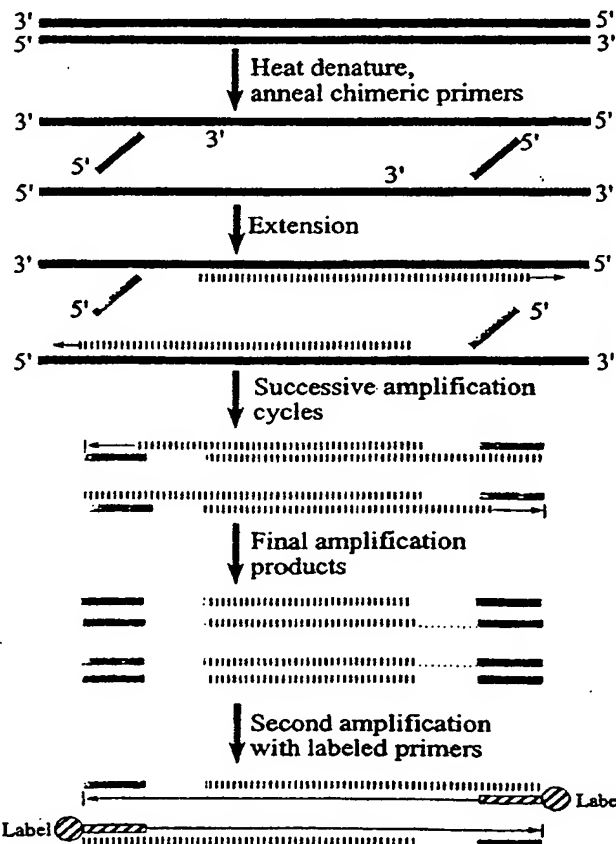
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68		A1	(11) International Publication Number: WO 99/58721
			(43) International Publication Date: 18 November 1999 (18.11.99)
(21) International Application Number: PCT/US99/10417		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 12 May 1999 (12.05.99)			
(30) Priority Data: 09/076,575 12 May 1998 (12.05.98) US			
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 09/076,575 (CON) Filed on 12 May 1998 (12.05.98)			
(71) Applicant (for all designated States except US): WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US).		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(72) Inventors; and			
(75) Inventors/Applicants (for US only): WANG, David, G. [CN/US]; 6 Hansel Road, Newtown, PA 18940 (US). LANDER, Eric, S. [US/US]; 151 Bishop Allen Drive, Cambridge, MA 02138 (US).			
(74) Agents: HOGLE, Doreen, M.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US) et al.			

(54) Title: MULTIPLEX DNA AMPLIFICATION USING CHIMERIC PRIMERS

(57) Abstract

The present invention provides for a method of simultaneously amplifying a large number of target sequences from a template nucleic acid and subsequently labeling the amplification products.



BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the P

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakhstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LJ	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

-1-

MULTIPLEX DNA AMPLIFICATION USING CHIMERIC PRIMERS

RELATED APPLICATIONS

This application is a Continuation of U.S. patent application serial number 09/076,575, filed May 12, 1998, the entire teachings of which are incorporated herein by reference.

GOVERNMENT SUPPORT

The invention was supported, in whole or in part, by grants HG00098 and HG01323 from the National Human Genome Research Institute, and grant 70NANB5H1031, from the National Institute of Standards and Technology. The United States Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

The Human Genome Project and associated research has produced a series of genetic maps of increasing density. Variation, i.e., polymorphism, is the foundation of any genetic map, and the types of polymorphisms used to build genetic maps have evolved over the years. Differences in gross morphology were used to construct the first genetic maps, but presented difficulties in that (1) morphological traits can be difficult to detect reliably, (2) there were few such reliable traits, which meant that very large populations had to be used to detect linkage between traits, and (3) similar morphology can be caused by several different

genes. In the 1970's, isozymes provided additional variation for constructing genetic maps. Restriction fragment length polymorphisms (RFLPs) and restriction site maps supplanted isozymes as the tools of choice in the 1980's. In the 1990's, simple sequence length polymorphisms (SSLPs, also called STRs, or Short Tandem Repeats) and sequence tagged sites (STSs) allowed construction of maps containing greater numbers of markers than ever before.

Single nucleotide polymorphisms (SNPs) are positions at which two alternative bases occur at appreciable frequency (>1%) in the human population, and are the most common type of human genetic variation. There is growing recognition that large collections of mapped SNPs would provide a powerful tool for human genetic studies. Because this type of variation is at the sequence level, it also opens a window on the root causes of variation, including differences in gross morphology and biochemistry, and susceptibility to genetic diseases. SNPs can also be used to create more markers for genetic maps, or to study linkage disequilibrium, or human evolution and migration. Before SNPs can be systematically applied in such studies, however, it is necessary to create a large collection of such loci, construct maps of their genomic locations, and develop methods for large-scale genotyping.

There have been many attempts to analyze large numbers of samples simultaneously, a method often referred to as "multiplexing." Attempts have been made to formulate such methods for high throughput sequencing (Church, G.M., and Kieffer-Higgins, S., Science 240:185-188 (1988)) and thermocycling (Shuber, A.P. et al., Genome Res. 5:488-493 (1995); Edwards, M.C., and Gibbs, R.A., PCR Meth. Appl. 3:S65-S75 (1994); Chamberlain, J.S. et al., Nuc. Acids Res. 16:11141-11156 (1988)), but they have either met with variable success, or have been

able to multiplex only a few samples at a time. With the discovery of increasing numbers of SNPs, there exists a real need to increase the efficiency with which they can be analyzed.

5 SUMMARY OF THE INVENTION

The present invention relates to a method of multiplex amplification by which a large number of target sequences on a template nucleic acid are amplified simultaneously, and labeled for detection.

10 During an amplification reaction, the primers are incorporated into the products. In the early cycles of the reaction, the primers copy the target sequences on the template nucleic acid. In later cycles of the reaction, however, the number of copies outnumbers (and
15 later overwhelms) the number of original template sequences. The primers begin to use the products from earlier cycles as template. As a result, the template/target sequence is copied, as is the primer that was incorporated into the 5' end of the
20 target/template during its production in the earlier cycle. The products from these later cycles (which at the end of the reaction form the vast majority of the products) consist of target sequence located between the incorporated primers at the 5' end, and the complements
25 of those primers at the 3' end.

The method of the invention makes use of chimeric primers, which are primers which have both a hybridization segment and a constant segment. The hybridization segment hybridizes to the template nucleic
30 acid so that extension by the polymerase can occur. The constant segment does not hybridize with the original template nucleic acid. As products from earlier cycles are used as templates, however, this constant segment also hybridizes to the template. This normalizes the
35 hybridization kinetics across the different target sequences being simultaneously amplified, preventing

loci from being over or underrepresented at the end of the reaction.

In general, the invention features a method for simultaneously amplifying a plurality of target
5 sequences from template nucleic acid and labeling the amplification products, comprising the steps of (a) combining template nucleic acid and a plurality of pairs of chimeric primers under conditions appropriate for members of the chimeric primer pairs to hybridize to
10 complementary nucleic acid sequences on the template nucleic acid sufficiently well to permit primer extension by a polymerase enzyme, thereby producing template-primer complexes, (b) subjecting the template-primer complexes to conditions appropriate for a first
15 amplification reaction, thereby producing a set of first amplification products, (c) combining these first amplification products and a plurality of pairs of labeled primers, under conditions appropriate for members of the labeled primer pairs to hybridize to
20 complementary nucleic acid sequences on the first amplification products sufficiently well to permit primer extension by a polymerase enzyme, thereby producing product-primer complexes, and (d) subjecting the product-primer complexes to conditions appropriate
25 for a second amplification reaction, thereby producing a set of second labeled amplification products. The template nucleic acid can be isolated nucleic acid, isolated genomic DNA, cDNA, or nucleic acid not isolated away from other cellular components. Each member of a
30 chimeric primer pair includes a hybridization segment and a constant segment. An example of a pair of constant segments is 5'-TAATACGACTCACTATAGGGAGA-3' (SEQ ID NO:1) for use on the forward primer and
5'-AATTAACCCTCACTAAAGGGAGA-3' (SEQ ID NO:2) for use on
35 the reverse primer. In the first amplification reaction (step (b)), the conditions include a high concentration of $MgCl_2$ (e.g., from about 2.5 millimolar to about 7.0

millimolar, or about 5 millimolar) and a low extension temperature (e.g., from about 60°C to about 70°C, from about 60°C to about 65°C, about 65°C). In the second amplification reaction (steps (c) and (d)), the labeled
5 primers can be labeled with a biotin molecule, a fluorophore, a dye, a metal, or a radionuclide. In addition, the labeled primers have constant segments identical to the constant segments of the chimeric primers used in the first amplification reaction.

10 In this embodiment, amplification and detection are performed in two separate reactions. The first reaction uses high levels of magnesium and low extension temperatures, and many (up to several hundred) pairs of chimeric primers, which are used to simultaneously
15 amplify several hundred target sequences on a template nucleic acid. Each chimeric primer includes a hybridization segment, which is nucleic acid which hybridizes to its complementary sequence on the template nucleic acid, and a constant segment, which is nucleic
20 acid which does not hybridize to the template nucleic acid. The hybridization segment is different for each chimeric primer and each pair of chimeric primers is designed so that the hybridization segment of each member of the pair hybridizes to a sequence on the
25 template nucleic acid which flanks a target sequence. That is, each pair of chimeric primers hybridizes to the template nucleic acid, in regions which flank the target (a portion of the template nucleic acid to be amplified); each member of a chimeric primer pair
30 hybridizes at one end of the target segment and, thus, together the pair of chimeric primers flank the target segment. Stated another way, each target sequence is amplified by its own unique pair of chimeric primers. For example, ten primer pairs (twenty primers, each with
35 a different hybridization segment), are needed to amplify ten different target sequences. The hybridization segments of the primers are therefore used

to amplify the target sequences on the template nucleic acid.

The first amplification reaction uses the chimeric primers to make copies of the target sequences. As these copies are made, the polymerase incorporates each chimeric primer into the copy being made.

A second amplification reaction follows, which labels the products from the first amplification reaction. This second reaction uses labeled primers which hybridize to the complements of the constant segments located at the 3' ends of the amplification products.

The constant segments of the chimeric primers (and therefore the labeled primers complementary to the products of those constant segments), are chosen so as to comprise sequence that is unrelated to the template nucleic acid, and is therefore unlikely to hybridize with it. For chimeric primers used to amplify mammalian DNA, for example, bacteriophage or insect sequences can serve as the constant segments. In one embodiment of the present invention, two constant segments derived from bacteriophage are used. One of the pair consists of the T7 promoter sequence 5'-TAATACGACTCACTATAGGGAGA-3' (SEQ ID NO:1), which is synthesized onto the 5' end of the 'forward' chimeric primer within each primer pair, and the other is the T3 promoter sequence 5'-AATTAACCCTCACTAAAGGGAGA-3' (SEQ ID NO:2), which is synthesized onto the 5' end of the 'reverse' primer. In this embodiment therefore, two different labeled primers are used. The first is a biotinylated T7 promoter sequence (5'-biotin-TAATACGACTCACTATAGGGAGA-3', SEQ ID NO:3), which hybridizes to the amplification products produced by the chimeric primer containing the T3 promoter sequence (SEQ ID NO:2). The second labeled primer is a biotinylated T3 promoter sequence (5'-biotin-AATTAACCCTCACTAAAGGG-3', SEQ ID NO:4), which hybridizes to the amplification

product produced by the T7 chimeric primer (SEQ ID NO:1). In other words, a single constant segment is used on all of the forward primers, and another constant segment is used on all of the reverse primers. These
5 constant segments normalize hybridization across all of the different loci, that is, they provide for similar hybridization kinetics for the many loci being amplified simultaneously. This results in roughly equal amounts of products from each locus. Use of the constant
10 segments provide an additional advantage in that they are used for detection in the second amplification reaction.

In another embodiment, a *single* sequence is used as the constant segment on all of the chimeric primers
15 (rather than two sequences as in the embodiment above). In such a case, the 'forward' and the 'reverse' chimeric primers have identical 5' tails, and a single labeled primer is, therefore, used to label the amplification products.

20 In either embodiment, the first amplification reaction amplifies the sequences on the template nucleic acid and the second amplification reaction labels the reaction products from the first amplification reaction. The combination of chimeric primers and reaction
25 conditions allows the *simultaneous* amplification of hundreds of loci. For example, as described herein, 558 loci have been successfully amplified with a 50% pass rate, 92 loci with an 85% pass rate, and 46 or 23 loci exhibited 90% and 92% pass rates, respectively. An
30 advantage of the invention is that these pass rates were achieved without further optimization. Alternatively, the conditions may be further optimized to achieve higher pass rates.

The invention also features a method for
35 simultaneously amplifying a plurality of target sequences from template nucleic acid and labeling the amplification products, including the steps of (a)

combining template nucleic acid and a plurality of pairs of labeled chimeric primers, under conditions appropriate for members of the labeled chimeric primer pairs to hybridize to complementary nucleic acid sequences on the template nucleic acid sufficiently well to permit primer extension by a polymerase enzyme, thereby producing template-primer complexes; and (b) subjecting the template-primer complexes to conditions appropriate for an amplification reaction, thereby producing a set of labeled amplification products. The amplification conditions may include a high concentration of $MgCl_2$ (e.g., from about 2.5 millimolar to about 7.0 millimolar, about 5 millimolar), and a low extension temperature (e.g., from about 60°C to about 70°C, from about 60°C to about 65°C, about 65°C). The template nucleic acid can be isolated nucleic acid, isolated genomic DNA, cDNA, or nucleic acid not isolated away from other cellular components. Each member of a labeled chimeric primer pair includes a hybridization segment and a labeled constant segment. For example, the sequence of the constant segment can be 5'-TAATACGACTCACTATAGGGAGA-3' (SEQ ID NO:1) for the forward primer and 5'-AATTAACCCTCACTAAAGGGAGA-3' (SEQ ID NO:2) for the reverse primer). The constant segment can be labeled with a biotin molecule, a fluorophore, a dye, a metal, or a radionuclide.

In another embodiment, amplification and labeling of the products are carried out in a single step. The chimeric primers can be synthesized as described above, and labeled (e.g., biotinylated) at the 5' end. In this embodiment, the reaction conditions are as for the first amplification reaction, that is, the conditions include high concentrations of $MgCl_2$ and low extension temperatures. The second amplification reaction is omitted.

In addition, the invention features a kit for simultaneously amplifying a plurality of target

sequences from a template nucleic acid and labeling the amplification products. The kit includes (a) a plurality of pairs of chimeric primers, and (b) at least one reaction mixture appropriate for use in

5 amplification reactions. At least one of the reaction mixtures includes a high concentration of $MgCl_2$ (e.g., from about 2.5 millimolar to about 7.0 millimolar, about 5 millimolar). Each member of a chimeric primer pair includes a hybridization segment and a constant segment

10 (e.g., 5'-TAATACGACTCACTATAGGGAGA-3' (SEQ ID NO:1) for the forward primer and 5'-AATTAACCCTCACTAAAGGGAGA-3' (SEQ ID NO:2) for the reverse primer). The constant segment may be labeled (e.g., with a biotin molecule, a fluorophore, a dye, a metal, or a radionuclide).

15 The invention also relates to kits containing the chimeric primer pairs and, optionally, reaction mixtures for practicing the method of the invention. Such kits can contain a collection of primer pairs useful to amplify a particular set of target sequences on a

20 template nucleic acid.

The invention has the advantage of allowing the simultaneous amplification of many (e.g., several hundred) target sequences in a single reaction and of allowing for labeling of the mixture of products. A

25 variety of methods are available for detecting and analyzing these products. Using size-based methods of detection (e.g., gels) can be difficult, due to the large number of different products that are created by the invention. The Examples below describe analysis by

30 means of genotyping chips, which is a non-size-dependent method of analysis.

The kit of the invention also has an advantage in that it contains a collection of primer pairs chosen so as to yield a particular type of information. For

35 example, the primer pairs can be chosen to detect susceptibility to a set of genetic diseases. For use in forensics studies, the primers can be selected to detect

polymorphisms in target sequences in highly variable regions of DNA. The polymorphisms found in an individual's DNA in those variable regions can be compared to the polymorphisms found in DNA from crime scene evidence in those same regions. If the polymorphisms from the individual and the evidence are different, then the individual is excluded from the pool of possible suspects. If they are the same, the individual cannot be excluded.

10 The methods and kits of the present invention can be used in humans and non-humans. For example, the methods, primers and kits can be used to assay polymorphisms in animals for veterinary purposes. For instance, sets of primers can be chosen to amplify target sequences known to be associated with susceptibilities to diseases with genetic components, or to detect known genetic defects in purebred animals such as dogs or horses. Primer sets can also be chosen to assess levels of biodiversity in populations of animals, plants, or microorganisms.

20 The methods and kits of the invention can also be used to amplify sequences across species. For instance, chimpanzees and humans share approximately 99% sequence similarity. The methods and kits of the invention can be used to locate those areas in which the 1% interspecific difference is located, thereby pinpointing the "evolutionary hotspots" responsible for species differentiation, and interspecific conserved regions, as well.

30 Kits can also be created to fingerprint proprietary biological material. For example, a set of primers can be chosen corresponding to specific genotypes known to exist in a protected crop cultivar. Assays of plants can be made according to the present invention, to determine if those plants correspond to the genotype of the patented cultivar.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred
5 embodiments of the invention, as illustrated in the accompanying drawings.

Figure 1 is a schematic diagram illustrating the use of chimeric primers in the amplification and labeling reactions, and the products made.

10 Figures 2A and 2B are more detailed diagrams and flow charts illustrating the same process as depicted in Figure 1. Figure 2A depicts the first amplification reaction, and Figure 2B depicts the second amplification reaction (the labeling reaction). "Target" indicates
15 the target sequence, whether on the template nucleic acid, or the amplification products. "zzzz" is meant to indicate nucleic acid sequence outside of the area of interest. In these diagrams, the forward chimeric primer has the sequence 5'-GGGTAAT-3', comprising the
20 hybridization segment "TAAT", and constant segment "GGG". The reverse primer (3'-GCCGTTT-5') has hybridization segment "CGGC" and constant segment "TTT". The labeled primers are 5'-biotin-GGG-3' (which hybridizes to the "CCC" amplified from (and therefore
25 complementary to) the forward chimeric primer), and 3'-TTT-biotin-5' (which hybridizes to the "AAA" amplified from (and therefore complementary to) the reverse chimeric primer).

Figure 3 is a diagram illustrating the relative
30 relationships on the template nucleic acid among the various target sequences, chimeric primers and primer pairs, and labeled primers of the method of the invention. "Target 1", "target 2", etc. denote the target sequences of SNP locus 1, 2, etc. "H1a", "H2a",
35 etc. are the hybridization segments for the forward primers for target 1, target 2, etc. "H1b", "H2b", etc. are the hybridization segments for the reverse primers

for target 1, target 2, etc. "Ca" is the constant segment for all of the forward chimeric primers, and "Cb" is the constant segment for all of the reverse chimeric primers.

5 DETAILED DESCRIPTION OF THE INVENTION

Described herein is a method of multiplex amplification. Using this method, several hundred pairs of chimeric primers are used to simultaneously amplify several hundred target sequences on template nucleic acid. In one embodiment of the method, two successive amplification reactions are performed. The first of the two amplification reactions uses high levels of magnesium, low extension temperatures, and pairs of chimeric primers to simultaneously amplify a large number (e.g., up to several hundred) target sequences on a template nucleic acid. By "high" levels of magnesium is meant at least 2.5 millimolar, preferably about 2.5 millimolar to about 7.0 millimolar, and more preferably about 5 millimolar. By a "low" extension temperature is meant, for a polymerase with a normally optimal extension temperature of 72°C, an extension temperature of about 60°C to about 70°C, preferably about 60°C to about 65°C, and most preferably about 65°C. The amplification enzyme should be subjected to a sub-optimal temperature during extension, so as to equalize amplification across all of the different primer parts in the reaction, and to prevent over-representation of the products of some loci over others. The chimeric primers used in the method each contain a hybridization segment and a constant segment. The hybridization segment hybridizes to the template nucleic acid in the vicinity of a target sequence; the constant segment consists of a sequence which does not hybridize to the template.

35 In this embodiment, a second amplification reaction follows, which uses labeled primers to detectably label

the reaction products of the first amplification reaction. The combination of chimeric primers and reaction conditions allows for the *simultaneous* amplification of a large number of loci. With these
5 methods, for example, 558 loci have been successfully amplified with a 50% pass rate, 92 loci have been amplified with an 85% pass rate, and 46 or 23 loci with 90% and 92% pass rates, respectively.

"Amplification" refers broadly to a process for
10 using a polymerase and a pair of primers for increasing the amount of a particular nucleic acid sequence, referred to as a target sequence, relative to the amount of that sequence initially present (on the template sequence). The process may be accomplished by the *in*
15 *vitro* methods of the polymerase chain reaction or ligase chain reaction, or others. A target sequence is a sequence that lies between the hybridization regions of two members of a pair of primers and is amplified by them. The target sequence generally exists as part of a
20 larger "template" sequence; however, in some cases, a target sequence and the template are the same. The template sequence may be an isolated nucleic acid or, alternatively, a nucleic acid which has not been separated away from the cellular components of the
25 biological source from which it was obtained. Although "template sequence" generally refers to the nucleic acid sequence initially present, the products from each amplification cycle are in fact used as template sequence in subsequent amplification cycles.

30 The template nucleic acid can be isolated by methods well known in the art. By "nucleic acid" is meant a length of DNA, RNA, cDNA, nucleic acids from mammals or other animals, plants, insects, bacteria, viruses, or other organisms. Nucleic acids referred to
35 herein as "isolated" are nucleic acids substantially free of (*i.e.*, separated away from) the cellular components of the biological source from which they were

obtained (e.g., as it exists in cells or in a mixture of nucleic acids such as a library), which may have undergone further processing. "Isolated" nucleic acids include nucleic acids obtained by methods described

5 herein, similar methods or other suitable methods, including essentially pure nucleic acids, nucleic acids produced by chemical synthesis, by combinations of biological and chemical methods, and recombinantly produced nucleic acids which are isolated (see e.g.,

10 Daugherty, B.L. et al., Nucleic Acids Res. 19(9):2471-2476 (1991); Lewis, A.P. and Crowe, J.S., Gene 101:297-302 (1991)). The template nucleic acid can therefore be a mixture of different nucleic acids or can be lengths of nucleic acid which are approximately the same (e.g.,

15 many copies of the same nucleic acid, such as nucleic acids which have been chemically synthesized or recombinantly produced). Alternatively, the template may occur as nucleic acid which has not been isolated from other cellular components, but has been treated in

20 such a way that it is available for hybridization with amplification primers. For example, cells containing the template DNA can be denatured at high temperature to break the cellular membranes and expose the nucleic acids, and the primers and amplification reaction

25 ingredients added directly to the denatured cellular "soup". Nucleic acids released in this way may be used in the method of the invention, although the success rate may not be as high as that reported herein by the Applicants.

30 A "primer" is a length of single-stranded nucleic acid, which is used in combination with a polymerase to amplify a region from a template nucleic acid. Primers are generally short (e.g., 15-30 bases), but can be longer if required. The primer must contain a sequence

35 which hybridizes with the template nucleic acid under the conditions used. Primers may be used singly, that is, a single primer consisting only of a single sequence

can be used in the amplification reaction, and will produce one copy of one strand of the template per cycle of amplification. This can be done in situations where a large number of copies is not required, or where only
5 one strand is to be copied (e.g., in producing antisense products), or if the sequence at the other end of the template is unsuitable for choosing a second primer.

More generally, a pair of primers is used in an amplification reaction. The two are of different
10 sequences, and are used in combination, and produce a copy of each template strand per cycle of amplification. The two different primers should not be complementary to each other, or they will hybridize to each other rather than the template, and the polymerase will then be
15 unable to make a copy of the template. Commonly, the two primers are chosen from sequence at the 5' end of each of the two complementary strands of the template nucleic acid.

A "chimeric primer pair" is a set of two chimeric
20 primers wherein the hybridization segments each hybridize to the template sequence on opposite flanks of the target sequence. A "chimeric primer" is a primer (e.g., a short piece of single-stranded nucleic acid) used for amplification of a target nucleic acid, wherein
25 the primer contains a sequence (i.e., the "hybridization segment") which hybridizes to the template nucleic acid in the vicinity of the target sequence, and a sequence which does not hybridize to the template nucleic acid. The method of the invention uses chimeric primers
30 comprising a hybridization segment and a constant segment. The constant segment should not hybridize to other constant segments, or to the hybridization segments of the chimeric primers. The constant segment is generally about 15 to about 35 base pairs, such as
35 about 20 to about 25 base pairs, about 15 to about 25 base pairs, about 15 to about 30 base pairs, about 20 to about 30 base pairs, about 15 to about 35 base pairs,

about 20 to about 35 base pairs, about 25 to about 35 base pairs, or about 30 to about 35 base pairs. The hybridization segments of the members of a given chimeric primer pair should have approximately the same T_m . That is, all of the segments that hybridize with the template will have melting temperatures within degrees of each other.

The hybridization segments are selected by methods commonly known in the art of nucleic acid amplification. That is, the hybridization segment is analogous to, and serves the same purpose as, the primer as it is commonly used in the art of nucleic acid amplification, and it is selected in the same way. To select a pair of primers (or in the present invention, a pair of hybridization sequences) for amplifying a particular target sequence, the sequence of the target nucleic acid must be approximately known (e.g., it has been sequenced in the organism being studied, or in a related organism). A short stretch of sequence at either end of the target is then selected to serve as the primer. The two primers which are intended to amplify a specific target are chosen on the basis of several characteristics, including length (e.g., 15-35 base pairs is common in the field), melting temperature, or other criteria. For instance, depending on the sequence at one end of the target, it may be necessary to choose a longer or shorter primer in order to obtain a melting temperature to match the primer at the other end of the target sequence. Such selection criteria are known in the field of nucleic acid amplification, and computer programs are available (e.g., PRIMER (Daly, M.J., Lincoln, S.E., and Lander, E.S., unpublished); Lerman, L.S., and Silverstein, K., Meth. Enzymol. 155:482-501 (1987)) which analyze sequence and choose candidate primers on the basis of specified parameters such as desired primer length and melting temperature.

The constant segments of the chimeric primer pairs are not selected to hybridize at the ends of the target nucleic acid. In fact, the constant segments are specifically selected so that they *not* hybridize with the template nucleic acid. In one embodiment, the method of the invention uses two constant segments, the first comprises the T7 promoter sequence (5'-TAATACGACTCACTATAGGGAGA-3', SEQ ID NO:1), which is synthesized onto the 5' end of the 'forward' primer.

The second constant segment comprises the T3 promoter sequence (5'-AATTAACCCTCACTAAAGGGAGA-3', SEQ ID NO:2), which is synthesized onto the 5' end of the reverse primer. Although these constant segments were chosen from T3 and T7 sequences, sequences from other organisms (e.g., insects, reptiles) might also prove useful, so long as those sequences generally lack the ability to hybridize with mammalian DNA. On the other hand, mammalian sequences might be used as the constant segments in situations where one wishes to construct chimeric primers to amplify non-mammalian template. There is no requirement that the constant segments have a complete lack of ability to hybridize to the template DNA, just that it tends not to hybridize with the template DNA in general.

Creation of an amplification product starts at the site where the primer hybridizes to the template nucleic acid, and during extension, the primer itself is incorporated into the product, resulting in a copy of the template nucleic acid which differs from the original in that it is truncated at the 5' end where the primer hybridized and extension began (Figure 1). As the amplification reaction progresses through its cycles, the amplification products begin to outnumber, and later overwhelm, the original template sequences.

Primers in later amplification cycles increasingly use the products from previous cycles as template, so that at the end of the reaction, the majority of the

amplification products consist of sequences which terminate at the 5' ends in the primer sequences (Figure 1 and Figures 2A and 2B (boxes)). The 3' ends of the products terminate with the complement of the chimeric primer (Figures 2A and 2B (dashed boxes)). The method of the invention uses chimeric primers with a non-hybridizing sequence at the 5' end, resulting in reaction products terminating in the constant sequences (Figure 2A, "Products after successive amplification cycles").

The method of the invention, in one of its embodiments, uses a second amplification reaction to label the reaction products of the first reaction (Figure 2A). Labeled primers are used in this reaction. A "labeled primer" is a primer (e.g., a short segment of single-stranded nucleic acid) which has a detectable label (e.g., biotin, fluorophore, radioactivity, heavy metals, dyes, etc.) attached to it. Two primers are used in this method: 5'-biotin-TAATACGACTCACTATAGGGAGA-3' (SEQ ID NO:3), and 5'-biotin-AATTAACCCTCACTAAAGGG-3' (SEQ ID NO:4). These hybridize with the constant segments of the chimeric primers, and the products of this second reaction also have the primers incorporated into them. These primers are labeled however, and the products are therefore detectable.

Figure 1 presents a simplified illustration of one embodiment of the method, which uses two separate amplification reactions. The first amplifies the targets. The products from this reaction are then detectably labeled in the second reaction. In Figures 2A and 2B, which illustrate the process in greater detail, the target is flanked by two sequences ("ATTA" and "CGGC") which are recognized by the hybridization segments on the chimeric primers ("TAAT" and "GCCG"). The constant segments of the chimeric primers ("GGG" and "TTT") do not hybridize with the template. The target

is copied from the 3' end of the chimeric primer. During the next cycle of amplification, this copy of the target also serves as a template. Unlike the original template nucleic acid however, this target/template is truncated at the 5' end, ending in the chimeric primer that was incorporated into the target/template during the previous cycle of amplification. These target/templates which are truncated at the 5' ends are then used as templates in the next cycle. The products of this next cycle are truncated at the 3' end, as well as the 5' end (Figure 2B).

Figure 3 illustrates the relationships on the template nucleic acid of the target loci, the chimeric primers, and the hybridization and constant segments. The template nucleic acid is depicted by the thin horizontal line, and the target loci (e.g., "target 1", "target 2", etc.) are symbolized by heavier bars on the template. "H" denotes a hybridization segment, and "C" a constant segment. "H1" and "C1" are the hybridization and constant segments for a chimeric primer intended to amplify "target 1", for example. The suffix "a" designates that the chimeric primer is the "forward" primer, and "b" indicates the "reverse" primer. From this figure, it can be seen that for x target loci, there will be 2x different hybridization segments, and therefore 2x different chimeric primers. Those 2x different chimeric primers have among them two different constant segments, however, one for the forward chimeric primer for each target locus, and another for the reverse.

The method of the invention uses high levels of magnesium and low extension temperatures for the first amplification reaction, and more standard conditions for the labeling reaction.

For the first reaction, a 50 μ l reaction volume should contain between approximately 20 and 200 ng of template nucleic acid (preferably 100 ng), about 0.1 to

about 1.0 μM of each chimeric primer each chimeric primer should be at about 0.1 to 1.0 micromolar concentration, (preferably 0.5 to 1.0 μM), about 1 unit of amplification enzyme, about 0.5 to about 2.0 mM dNTPs (preferably 1 mM), about 10 mM Tris-HCl (pH 8.3), about 5 mM KCl, about 2.5 to about 7.0 mM MgCl_2 (preferably 5 mM), and about 0.001% gelatin.

The hybridization segments of the chimeric primers are selected so as to have a T_m (melting temperature) of about 55°C to about 60°C, with a preferred T_m of about 57°C. During the first amplification reaction, an annealing temperature of about 50°C to about 57°C is preferred, and the extension temperature should be about 70°C to about 60°C, preferably about 65°C.

In the second amplification reaction, the ingredients are at concentrations commonly used by those of skill in the art, and the temperature conditions are likewise as those commonly used.

In another embodiment of the invention, the second amplification reaction could be omitted if the chimeric primers themselves were labeled (i.e., if each chimeric primer comprised a hybridization segment and a labeled constant segment). If the constant segments of the chimeric primers were labeled (if the 5' ends of the chimeric primers were biotinylated, for example), then the first amplification reaction would simply create labeled amplification products. Extrapolating from the embodiment described above, for example, such a labeled constant segment could comprise the biotinylated T7 promoter sequence (5'-biotin-TAATACGACTCACTATAGGGAGA-3', SEQ ID NO:3), added to the 5' end of the hybridization segment of the 'forward' primer. The second constant segment would comprise a labeled T3 promoter sequence (5'-biotin-AATTAACCCTCACTAAAGGGAGA-3', SEQ ID NO:4), added to the 5' end of the reverse primer. To ensure stability of hybridization, it might be necessary to

increase or decrease the length of the constant segment in some cases.

One of skill in the art will recognize that there exist a variety of detection schemes that can be used with the Applicants' method, including chemiluminescence, radioactivity, fluorophores, dyes, heavy metals, or staining. There are also a wide number of substitutions that can be made and alternative ways of practicing the Applicants' method. These alternatives are known to those of skill in the art from reading the scientific literature and are also available in compendiums of common laboratory procedures (e.g., Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York; Ausubel, F.M. et al., eds., *Current Protocols in Molecular Biology*; Erlich, H.A., ed., *PCR Technology*, Stockton Press, New York (1989). All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

Another method of practicing the invention is to use a single constant segment, instead of the two segments described above and illustrated in Figures 2A, 2B and 3. In Figures 2A and 2B, such a change would be shown by all of the constant segments having the same sequence (e.g., "GGG" or "TTT" instead of "GGG" and "TTT"), and in Figure 3, all the constant segments would simply be labeled "C" (not "Ca" and "Cb").

When multiplexing (amplifying many loci simultaneously), it is imperative to be able to correctly differentiate between the amplification products from different loci. There are two commonly-used methods of accomplishing this; (1) size discrimination, or (2) on the basis of differentially labeling the reaction products. The first method is most commonly done with detection methods which separate on the basis of size (e.g., gels). One chooses loci to

amplify together based on the ability of those loci to create products of different sizes, e.g., locus 1 creates a product of 100 bp, locus 2 creates a 200 bp, etc. Choosing which loci to amplify together is labor-intensive and requires careful planning. It is also possible that new, previously uncharacterized alleles that appear in the "zone" of another locus' reaction products may be misassociated with that other locus. Size-based analysis methods are therefore limited by the number of loci that can be unambiguously separated in the space offered by the separation method (e.g., the length of the gel). The second method, differentially labeling the reaction products, generally involves the attachment of different tags onto the primers for different loci. Fluorescent tags are the most commonly used because one can use molecules which fluoresce at different wavelengths, with the result that amplification products from different loci fluoresce in different colors. This method is limited, however, by the number of different fluorescent molecules available on the market. Both of these methods impose inherent limitations on the number of loci that can be multiplexed together. Another possibility is to use a method modified from that described by Church and Kieffer-Higgins (Science 240:185-188 (1988), wherein the amplification products are separated on a gel, which is then blotted, and successively probed with radiolabelled versions of the amplification primers. This method is also labor-intensive, in addition to being slow.

"Genotyping chips" can be used to detect, and differentiate between, the amplification products of the different loci. A "genotyping chip" is a high-density array of oligonucleotide probes to which a sample is hybridized. Hybridization to one of the oligonucleotides indicates that the sample is positive for that oligonucleotide sequence (Fodor, S.P.A. et al., Science 251:767-773 (1991)). If aligned, overlapping

sequences are arrayed (e.g., ABC, BCD, CDE, etc.), then the chip can be used for sequencing (Pease, A.C. et al., Proc. Natl. Acad. Sci. USA 91:5022-5026 (1994)).

Alternatively, the chip can contain oligonucleotides
5 corresponding to polymorphic alleles (Chee, M. et al., Science 274:610-614 (1996)). If there are ten different alleles at a particular locus, for instance, then oligonucleotides corresponding to those ten alleles can be placed on a genotyping chip. Hybridization to one of
10 the alleles indicates that the sample is positive for that allele (see Example 2, "Design of Genotyping Chips," *infra*).

In the method of the invention, the hybridization segments of the chimeric primers are chosen so as to
15 produce relatively short target sequences (e.g., 70-80 base pairs). Amplification products of such similar lengths would be extremely difficult to differentiate by size separation methods (e.g., electrophoretic gels), but are well-suited to detection by genotyping chips, or
20 other size-insensitive detection methods.

The method of the invention also allows for the simultaneous detection of many polymorphisms in a sample. A "polymorphism" is, fundamentally, an allelic variation between the nucleic acids of two samples.
25 Such variations can range from gross morphological differences to differences in biochemistry, conformation of biomolecules, or differences in nucleic acid sequences. The "samples" being examined can be whole organisms, or portions thereof, and can represent single
30 individuals, or pooled populations. Polymorphisms include differences in nucleotide sequence, mutations, insertions, deletions, point mutations, or structural differences, as well as strand breaks or chemical modifications that result in an allelic variant in the
35 form of a mismatch. A polymorphism between two nucleic acids can occur naturally, or be caused by exposure to or contact with chemicals, enzymes, or other agents, or

can be caused by circumstances which cause damage to nucleic acids (e.g., exposure to ultraviolet radiation, mutagens or carcinogens).

The examples below illustrate use of the method of the invention to detect single nucleotide polymorphisms, or "SNPs," which are polymorphisms that consist of a difference in a single nucleotide. However, two sequences being compared may differ at more than a single nucleotide position. If the second difference occurs within the region where a primer should hybridize, that primer will not hybridize, and no reaction will occur. If the two differences both occur within the region that is amplified, aberrant patterns will be seen during the detection. In either case, it will be clear that the locus must be investigated further.

Multiplex amplification of large collections of mapped SNPs will provide a powerful tool for genetic studies, some of which are described below.

20 Linkage Mapping

Any type of DNA polymorphism can be used to trace inheritance of disease genes in pedigrees (Lander, E.S. and Schork, N., Science 265:2037-2048 (1994)). For technical reasons, geneticists had primarily employed restriction fragment length polymorphisms (RFLPs) during the 1980s and simple sequence length polymorphisms (SSLPs or microsatellites) during the 1990s. Both of these methods assay length differences by gel electrophoresis. SNPs are now being seen as an attractive alternative for the future, because there exist a variety of assays that may allow greater automation, parallelism, and throughput than can be achieved with length measurement (Conner, B.J. et al., Proc. Natl. Acad. Sci. USA 80:278-282 (1983); Cronin, M.T. et al., Hum. Mutation 7:244-255 (1996)). SNPs have only two alleles and thus are less informative than

typical multi-allelic SSLPs, but this deficiency can be offset by greater density: a genome scan with 1,000 well-spaced SNPs will extract roughly the same linkage information as the current standard of 400 well-spaced
5 SSLPs (Kruglyak, L., Nat. Genet. 17:21-24 (1997)). In addition, as shown below, SNPs can be assayed more efficiently than SSLPs, thereby providing a true advance over SSLP methodology.

Linkage Disequilibrium Mapping

10 Linkage disequilibrium mapping extends genetic analysis from families to populations by using high density genetic maps to recognize chromosomal segments descended from a common ancestor (Lander, E.S. & Botstein, D., Cold Spring Harbor Symp. Quant. Biol.
15 51:49-61 (1986)). Such analyses are useful in determining the location of a chromosomal segment conferring disease susceptibility. Ancestral segments that occur significantly more often in affected individuals are likely to harbor a susceptibility
20 allele. The precise SNP density required to detect linkage disequilibrium varies with the age and structure of the population.

Association Studies

Beyond simply serving as chromosomal markers, some
25 SNPs are actually the cause of functional variation in a gene. Several authors have suggested cataloguing coding-region SNPs (cSNPs) in all 100,000 human genes and performing association studies between these SNPs and different phenotypic traits (Risch, N. & Merikangas, K., Science 273:1516-1517 (1996); Lander E.S., Science 274:536-539 (1996); Collins, F.S., Guyer, M.S. and Chakravarti, A., Science 278:1580-1581 (1997)). For
30 example, the association between the ApoE gene and Alzheimer's disease, or between the Factor V gene and
35 deep vein thrombosis could have been discovered in this

fashion (Strittmatter, W.J. and Roses, A.D., Ann. Rev. Neurosci. 19:53-77 (1996); Voorberg, J., Lancet 343:1535-1536 (1994)).

Other Applications

5 The invention has the advantage of allowing the simultaneous amplification of many (e.g., several hundred) target sequences in a single reaction, and of allowing for labeling of the mixture of products.

10 Kits can be made for use with the method of the invention. Such kits would contain pairs of chimeric primers intended to label specific target sequences on the template nucleic acid of an organisms or organisms, and also labeled primer pairs to label the amplification products. If the chimeric primers in the kit have a
15 single constant segment (rather than two; i.e., one for the forward primer, and one for the reverse), then only a single labeled primer need be included. Components for the two different amplification reactions may also be included (e.g., amplification reaction buffers,
20 polymerase, etc.). If the amplification and labeling are done in a single reaction, then reaction components for only that single reaction need be included.

 While the Examples below demonstrate use of the methods and kits of the invention in the detection of
25 SNPs, the methods are not limited to this use. The methods and kits of the invention can be used in any situation where it is desirable to amplify a large number of target sequences, regardless of whether or not they contain a polymorphism. For instance, primers can
30 be chosen to amplify regions thought to be conserved, in order to rapidly identify individuals polymorphic in those regions. The methods and kits of the invention can also be used to evaluate interspecific polymorphism (such as between humans and chimpanzees) to locate both
35 conserved regions and also "hotspots" of evolutionary change.

The method and kits of the invention can be used for forensic identification of individuals. Primer pairs can be selected to detect polymorphisms in target sequences in highly variable regions of DNA. The

5 polymorphisms found in a suspect's DNA in those variable regions can be compared to the polymorphisms found in DNA from crime scene evidence in those same regions. Matching polymorphisms includes the suspect in the pool of possible perpetrators, while differing polymorphisms

10 excludes the suspect. These same sets of primers can also be useful in "biometrics" applications, which is the identification and verification of individuals via a unique biological profile. Potential applications of biometrics include the identification of deceased

15 persons, verification of identities of prisoners slated for release, or verification of a person's access to sensitive information or areas.

The kit of the invention has an advantage in that it can be assembled to contain a collection of primer

20 pairs chosen so as to yield a particular type of information. For example, the primer pairs can be chosen to detect susceptibility to a genetic disease or set of diseases, or the presence of pathogens or parasites. Chromosomal deletions known to lead to

25 cancer or other diseases can also be detected in this manner.

The methods and kits of the present invention can be used in humans and non-humans. For example, the methods, primers and kits can be used to assay sequences

30 in animals for veterinary purposes (e.g., presence of pathogens or parasites). Sets of primers can be chosen to amplify target sequences known to be associated with susceptibilities to diseases with genetic components, or to detect known genetic defects in purebred animals such

35 as dogs or horses.

The primer sets in the kits can also be chosen to assess levels of biodiversity in field populations of

animals, plants, or microorganisms. Individual organisms can also be "fingerprinted" and later re-identified, e.g., in animal migration studies, for instance. These same kits can also be used to study the evolution and migration of animal and plant populations.

Kits can also be created to fingerprint proprietary biological material, such as microbiological strains, crop cultivars, or animals. For example, a set of primers can be chosen corresponding to highly variable regions known to exist in a protected crop cultivar. Assays of plants can be made according to the present invention, to create unique genetic profiles, and to determine if those plants correspond to the genotype of the patented cultivar. Parentage of purebred animals can also be verified in this way.

The invention is further described in the following examples, which are intended to illustrate, and not limit, the scope of the invention described therein and in the claims.

20 **EXAMPLES**

Methods

Individuals Surveyed for SNPs

The individuals surveyed from chosen from CEPH pedigrees K104, K884 and K1331, from the Amish, Venezuelan and Utah populations, respectively. The SNP survey by gel-based sequencing examined three unrelated individuals (K104-1, K884-2, K1331-1) and a pool of ten individuals (K104-13, -14, -15, -16; K884-15, -16; K1331-12, -13, -14, -15).

30 **DNA Sequencing of SNPs from Sequence Tagged Sites (STSs)**

STSs were amplified with their corresponding amplification primers as described in Hudson, T.J. et al. (Science 270:1945-1954 (1995)) and Dietrich et al. (Dietrich, W. F. et al., Nature 380:149-152 (1996); Dietrich, W. F. et al., Nature Genetics 7:220-245;

Dietrich, W. et al., Genetics 131:423-447 (1992)), which are herein incorporated by reference in their entirety. The forward primer was modified to include the M13 -21 primer site 5'-TGTAACGACGCGCCAGT-3' (SEQ ID NO:5) at its 5'-end. The resulting amplification products were subjected to dye-primer sequencing (Koontz, W.L.G. and Fukunaga, K., IEEE Trans. Comp., C-21,171-178 (1972), herein incorporated by reference in their entirety), with products detected on an ABI377 or ABI373 fluorescence sequence detector. Possible sequence variations were detected by the ABI *Sequence Navigator* software package, which suggests potential heterozygotes by identifying nucleotide positions at which a secondary peak exceeds a selected threshold of 50%. Such apparent variations were then visually inspected, to compare the patterns seen among the several individuals.

Example 1: Obtaining SNPs

A: SNPs from Sequence Tagged Sites (STSS)

SNPs were obtained by surveying sequence-tagged sites (STSS) distributed across the human genome. These STSS are short genomic sequences, each with a corresponding amplification assay. An initial collection of 1,139 STSS was chosen from among the 24,568 STSS that had been used in the construction of a physical map of the human genome at the Whitehead/MIT Center for Genome Research (Hudson, T.J. et al., Science 270:1945-1954 (1995) and Schuler, G. et al., Science 274:540-546 (1996), herein incorporated by reference in their entirety). These STSS contain a total of 279 kb of genomic sequence between all of the amplification primer sites, with one-third consisting of random genomic sequence and two-thirds of 3'-ends of expressed sequence tags (3'-ESTs) and primarily representing untranslated regions of genes.

Each STS was amplified from four samples: three of the samples were individual human DNAs and the fourth

was pooled DNA from ten individuals. The amplification products were subjected to single-pass DNA sequencing using fluorescent-dye primers and gel electrophoresis; sequence traces were compared by a computer program followed by visual inspection (See "Methods," *supra*). Candidate SNPs were declared when two alleles were seen among the three individuals, with both alleles present at a frequency greater than 30% in the pooled sample. The term 'candidate SNP' is used because a subset of such apparent polymorphisms turn out to be sequencing artifacts, as discussed below in Example 3, *infra*.

The survey found 279 candidate SNPs, corresponding to a rate of one SNP per 1001 base pairs screened and an observed nucleotide heterozygosity of 3.96×10^{-4} (Table 1). The SNPs were distributed among 239 STSs, with some containing more than one polymorphic site. The polymorphism rate was lower in 3'-ESTs than in random genomic sequence, although the difference fell just short of statistical significance ($p = 0.057$, one-sided). The ratio of transitions to transversions was 2:1. Although the dinucleotide CpG comprises only about 2% of the sequence surveyed, nearly 25% of the SNPs occurred at such sites with the substitution nearly always being C \leftrightarrow T. Cytosine residues within CpG dinucleotides are well known to be the most mutable sites within the human genome, because most are methylated and can spontaneously deaminate to yield a thymidine residue (Cooper, D.N. & Karwczak, M., Hum. Genet. 85:55-74 (1990)). In addition to the single base substitutions, 23 insertion/deletion polymorphisms were also found (with all but eight involving a single base), corresponding to a frequency of one per 12 kb surveyed.

Table 1: Results of SNP screening

	Gel-based Sequencing			Chip-Based Detection		
	All STSs	3'-ESTs	random genomic STSs	All STSs	3'-ESTs	random genomic STSs
No. STSs screened	1,139	705	434	16,725	12,649	4,076
Total bases screened	279,165	186,524	92,641	1,981,030	1,324,320	656,710
No. candidate SNPs found	279	161	118	2,748	1,749	999
SNP frequency	1/1001	1/1159	1/785	1/721	1/757	1/657
Heterozygosity (x10 ⁻⁴)	3.96 ± 0.38	3.42 ± 0.43	5.04 ± 0.67	4.58 ± 0.15	4.36 ± 0.18	5.02 ± 0.28
No. STSs containing SNPs	239	137	102	2,299	1,515	784
% transitions among SNPs	67%	67%	67%	70%	70%	71%
% SNPs occurring within CpG	24%	23%	25%	24%	25%	22%

B: SNPs from other sources

Additional SNPs were isolated by using two directed approaches using public databases. First, reports in the literature of common variants in gene coding regions were collected. Out of 143 cases tested, 121 variants were confirmed by the detection of two alleles in the four-sample screening panel described above. The remaining 22 reported variants may be true polymorphisms, but simply monomorphic in the individuals tested. The second approach utilized the GenBank database, which contains multiple entries for some ESTS. Such entries were compared to identify single-nucleotide differences, which might reflect either common polymorphisms or sequencing errors in single-pass EST

sequencing. 200 such apparent differences were tested and the presence of a SNP was confirmed in 93 cases. These two directed approaches thus yielded an additional 214 SNPs.

5 Example 2: Design of Genotyping Chips

Gel-based sequencing was satisfactory for the initial screen (Example 1, above), but a more streamlined approach is needed in a large-scale survey. High-density DNA probe arrays provide an alternative approach for analyzing DNA sequences (Chee, M. et al., Science 274:610-614 (1996); Kozal, M.J. et al., Nat. Medicine 2:753-759 (1996)). Such 'DNA chips' can be made by using parallel light-directed chemistry to synthesize specified oligonucleotide probes covalently bound at defined locations on a glass surface or 'chip' (Fodor, S.P.A. et al., Science 251:767-773 (1991); Pease, A.C. et al., Proc. Natl. Acad. Sci. USA 91:5022-5026 (1994)). Current technology allows fabrication of 1.28 cm x 1.28 cm arrays of ~320,000 distinct oligonucleotides, each residing in a 'feature' of ~20 x 25 microns and containing more than 10⁷ copies of the oligonucleotide.

A target DNA sequence of length L can be screened for polymorphism by hybridizing a biotin-labeled sample to a variant detector array (VDA) of size $8 \times L$. For each position on both strands, the array has four 25-mer probes complementary to the sequence centered at the position. The four differ only in that the central (13th) position is substituted by each of the four nucleotides. Individuals that are homozygous for the expected sequence (e.g., "A-A") should hybridize more strongly to the perfectly complementary probe than to the three probes containing a central mismatch. The presence of an SNP would be expected to give rise to a different hybridization pattern, with homozygotes showing strong hybridization to an alternative base

(e.g., "G-G") and heterozygotes (e.g., "A-G") showing hybridization to two probes (e.g., "A" and "G"). The VDA thus signals the presence of a sequence variant and, in many cases, indicates the nature of the change.

5 VDAs have been used previously for mutation detection of small, well-studied DNA targets in large numbers of samples, including 387 base pairs from the HIV-1 genome, 3.5 kb from the BRCA1 gene, and 16.6 kb from the human mitochondrion (Chee, M. *et al.*, Science
10 274:610-614 (1996); Kozal, M.J. *et al.*, Nat. Medicine 2:753-759 (1996); Hacia, J.G. *et al.*, Nat. Genet. 14:441-447 (1996)). In this setting, the normal hybridization pattern can be characterized with great precision and mutations detected with high accuracy.

15 The current project, however, sought to use VDAs in a large-scale survey. A total of 16,725 STSs covering 2 Mb of human DNA was selected, one-third from random genomic sequence and two-thirds from 3'-ESTs. The survey employed 149 distinct chip designs, each
20 containing 150,000-300,000 features.

The STSs were examined in seven unrelated individuals (K104-1, -16, K884-2, -15, -16, K1331-12, -13), chosen from the CEPH pedigrees K104 (Amish), K884 (Venezuelan), and K1331 (Utah). Altogether, this
25 represented a survey of about 14 Mb of genomic sequence. For each chip, the corresponding STSs were amplified from an individual, pooled together, biotin-labeled, hybridized and stained as follows.

STSs were amplified with their corresponding
30 primers as described in Hudson *et al.* (Science 270:1945-1954 (1995)). Amplification products intended for hybridization to the same chip (typically amplification products from 100-200 STSs from a single individual) were pooled together for subsequent
35 processing. Approximately 1-2 μ g of the pooled amplification product was purified with Qiaquick purification kit (Qiagen, Hilden, Germany), fragmented

with DNase I, then biotin-labeled with terminal deoxynucleotidyl transferase (TdT, Life Technologies, GibcoBRL, (Gaithersburg, Maryland), according to the manufacturer's instructions. The fragmentation was

5 performed in a 40 μ l reaction with 0.2 unit of DNase I (Promega, Madison, Wisconsin), 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, and 50 mM potassium acetate at 37°C for 15 minutes, after which the reaction was stopped by heat inactivation at 96°C for 15 minutes.

10 The terminal transferase reaction was performed by adding 15 units of TdT and 12.5 mM biotin-N6-ddATP (DuPont-NEN Products, Boston, MA) to the preceding reaction mixture, incubating at 37°C for 1 hour and then heat-inactivating at 96°C for 15 minutes.

15 The labeled samples were hybridized to the chip as follows. Samples were denatured at 96°C for 5-6 minutes and cooled on ice for 2-5 minutes. Chips were pre-hybridized with 6X SSPET (0.9 M NaCl, 60 mM NaH₂PO₄, 6 mM EDTA (pH 7.4), 0.005% Triton X-100) for

20 approximately 5 minutes and then hybridized with the denatured sample in hybridization buffer (3M tetramethylammonium chloride, 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.01% Triton X-100, 100 mg/ml herring sperm DNA, and 200 pM control oligonucleotide) at 44°C for 15

25 hours on a rotisserie at 40 RPM. Chips were washed 3 times with 1X SSPET and 10 times with 6X SSPET at 22°C, then stained at room temperature with staining solution (2 mg/ml streptavidin R-phycoerythrin (Molecular Probes, Eugene, Oregon) and 0.5 mg/ml acetylated BSA in 6X

30 SSPET) for 8 minutes. After staining, chips were washed 10 times with 6X SSPET at 22°C on a fluidics workstation (Affymetrix, Santa Clara, California). Hybridization to the chip was detected by using a confocal chip scanner (HP/Affymetrix, Santa Clara, California) with a

35 resolution of 40-80 pixels per feature and a 560 nm filter, and visual inspection.

At each position, samples were classified as homozygous for the expected sequence, homozygous for an alternative sequence, or heterozygous. A collection of 2,748 candidate SNPs was identified, corresponding to a
5 rate of one per 721 base pairs surveyed and an observed nucleotide heterozygosity of 4.58×10^{-4} (Table 1, *supra*). The number of STSs containing SNPs was 2,299. The SNPs had a mean heterozygosity of 33%, with the minor allele having a mean frequency of 25%. SNPs were
10 found less often in 3'-ESTs than in random genomic sequence ($p < 0.023$, one-sided).

The nucleotide heterozygosity rate was indistinguishable from the estimate obtained from gel-based sequencing in Example 1, above ($p > 0.12$,
15 two-sided test). The ratio of transitions to transversions and the proportion of SNPs occurring at CPG dinucleotides were also indistinguishable. The frequency of SNPs was higher in the chip-based survey because more samples (14 vs. 6 haploid genomes) were
20 surveyed.

Example 3: Evaluation of Accuracy of Gel-based vs. Chip-based Surveys

Large-scale surveys are generally imperfect, and it is therefore important to assess the error rates of the
25 two systems. False positive rates were estimated by retesting candidate SNPs by thorough multipass gel-based sequencing. In the single-pass gel-based sequencing, 16% of the 120 candidate SNPs proved to be false positives, compared to 12% of the 220 candidate SNPs
30 found in the chip-based survey. False negative rates were estimated by including a subset of STSs in both surveys. Fifty-five SNPs were identified with both of the two survey methods, and were carefully confirmed to eliminate false positives. Eight (15%) of the 55 were
35 missed by single-pass gel-based resequencing and seven (13%) missed by the chip-based survey. Many of the

errors were due to random factors, because they were eliminated simply by repeating the original experiment. However, some errors were reproducible artifacts that could be eliminated only by changing the detection
5 protocol (for example, by using dye-terminators rather than dye-primers in gel-based sequencing). The rates were broadly similar in the two surveys, corresponding to roughly one false positive and false negative every 6,000-10,000 bases.

10 The error rates reflect the particular implementation of the large-scale survey (single-pass coverage for gel-based sequencing; one-color hybridization at one temperature to a single VDA design), rather than inherent limitations of the
15 technologies. It is likely that at the expense of additional effort, both technologies can provide higher accuracy still in future large-scale screens.

With current limitations, candidate SNPs should be confirmed before being regarded as certain. We
20 initially confirmed SNPs by using gel-based resequencing, but subsequently developed an accurate chip-based method using genotyping chips (Example 4, below).

Example 4: Genotyping Chips for Multiplex Amplification

25 Efficient methods are required for large-scale genotyping of SNPs, and one approach is to extend the use of chip-based re-sequencing from SNP discovery to SNP genotyping (Cronin, M.T. et al., Hum. Mutation 7:244-255 (1996)).

30 Genotyping chips containing 'genotyping arrays' were synthesized for each SNP to be tested. Each genotyping array consists of two short VDAs corresponding to the two alternative alleles. The presence of an allele is reflected in strong
35 hybridization to the corresponding resequencing array. Amplification assays were designed for the region

containing each SNP, with the goal of being robust and mutually compatible. This was done by ensuring that (1) the amplification targets were small (typically a few nucleotides around the polymorphic site), (2) the
5 primers all had similar calculated melting temperatures, and (3) constant sequences were added to the 5'-ends of the forward and reverse primers to facilitate batch-labeling of pooled amplification products. Each assay was tested to ensure that it amplified a single
10 fragment from genomic DNA.

For each SNP, primers were chosen using the PRIMER software package (Hudson, T.J. et al., Science 270:1945-1954 (1995)) to closely flank the polymorphic base and to have a predicted melting temperature of
15 57°C. Forward and reverse primers were synthesized with the T7 (5'-TAATACGACTCACTATAGGGAGA-3', SEQ ID NO:1) and T3 (5'-AATTAACCTCACTAAAGGGAGA-3', SEQ ID NO:2) promoter sites at their respective 5'-ends. Each primer pair was individually tested to determine if it produced a single
20 clear fragment visible by agarose gel electrophoresis and ethidium bromide staining, as described in Hudson et al. (Science 270:1945-1954 (1995)). Amplification assays passing this test were further classified as being "strong" or "weak," according to the yield of the
25 fragment produced. Primer pairs were grouped into multiplex sets, with the sets chosen to consist of either all strong assays or all weak assays.

The most complex genotyping chip tested contained genotyping arrays for 558 candidate SNPs identified in
30 the chip-based survey. Initially, the 558 loci were separately amplified, then pooled, labeled and hybridized to the chip. To determine whether each locus could be reliably read, a formal 'detection test' was defined: a locus passed if, for each of three
35 individuals tested, the expected DNA sequence could be successfully read on both strands for one or both alleles. In all, 98% of the loci passed this detection

test, with the remaining ten failing due to weak hybridization or cross-hybridization.

Example 5: Multiplex Amplification

In multiplex amplification, primer pairs from many
5 different loci are combined in a single reaction and
amplified simultaneously. Specifically, multiplex
amplification reactions were performed in a 50 μ l volume
containing 100 ng of human genomic DNA, 0.5-1.0 μ M of
each primer, 1 unit of AmpliTaq Gold (Perkin-Elmer,
10 Foster City, California), 1 mM dNTPs, 10mM Tris/HCl (pH
8.3), 50 mM KCl, 5 mM MgCl₂, and 0.001% gelatin.
Thermocycling was performed on a Tetrad (MJ Research,
Watertown, Massachusetts), with initial denaturation at
96°C for 10 minutes followed by 30 cycles of
15 denaturation at 96°C for 30 seconds, primer annealing at
55°C for 2 minutes, and primer extension at 65°C for 2
minutes. After 30 cycles, a final extension reaction
was carried out at 65°C for 5 minutes. Because the
resulting amplification products were small, it was
20 unnecessary to fragment them (as was done for the STSs
in the SNP screen in Example 2, *supra*). The products
were then biotin-labeled in a standard amplification
reaction, by using T7 and T3 primers with biotin-labels
at their 5'-ends (5'-biotin-TAATACGACTCACTATAGGGAGA-3'
25 (SEQ ID NO:3), and 5'-biotin-AATTAACCCTCACTAAAGGG-3'
(SEQ ID NO:4), respectively). The reaction was
performed with 1 μ l of template DNA, 0.5-1.0 μ M of
labeled primer, 1 unit of AmpliTaq Gold (Perkin-Elmer,
Foster City, California), 100 μ M dNTPs, 10mM Tris-HCl
30 (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 0.001% Gelatin.
Thermocycling was performed with initial denaturation at
96°C for 10 minutes followed by 25 cycles of
denaturation at 96°C for 30 seconds, primer annealing at
52°C for 1 minute, and primer extension at 72°C for 1
35 minute. After 25 cycles, a final extension reaction was
carried out at 72°C for 5 minutes. The amplification

products from the various multiplex reactions for an individual were then pooled together. One-tenth of the pooled sample was denatured and used for chip hybridization. Chips were hybridized, washed, stained and scanned, as described in Example 2, *supra*.

Although it is typically difficult to combine many thermocycling assays, this approach worked surprisingly well: of the 558 loci, 92% passed the detection test when amplification was performed in 24 sets of ~23 loci; 90% passed when amplified in 12 sets of ~46 loci; 85% passed when amplified in 6 sets of ~92 loci; and 50% passed when amplified in a single set of 558 loci. The success appears to have resulted from a combination of factors, including the small size of the amplification targets, optimization of amplification conditions and the presence of the constant sequence at the 5'-ends of the primers. Unsuccessful assays can be salvaged by grouping them into additional multiplex sets or by redesigning the assays.

Multiplex amplification of sets of 46 loci were used in subsequent experiments, because the number of reactions was decreased by 46-fold while allowing the vast majority of loci (512 out of 558) to be assayed. The procedure was further tested in 39 individuals and proved quite consistent: 96% of the 572 loci could be successfully read in 100% of individuals tested. The remaining 4% of the loci were successfully read in nearly all of the individuals.

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to

ascertain using no more than routine experimentation,
many equivalents to the specific embodiments of the
invention described specifically herein. Such
equivalents are intended to be encompassed in the scope
5 of the claims.

CLAIMS

What is claimed is:

1. A method for simultaneously amplifying a plurality
of target sequences from template nucleic acid and
5 labeling the amplification products, the method
comprising:
 - (a) combining template nucleic acid and a
plurality of pairs of chimeric primers,
wherein each member of a chimeric primer pair
10 comprises a hybridization segment and a
constant segment, under conditions appropriate
for members of the chimeric primer pairs to
hybridize to complementary nucleic acid
sequences on the template nucleic acid
15 sufficiently well to permit primer extension
by a polymerase enzyme, thereby producing
template-primer complexes;
 - (b) subjecting the template-primer complexes to
conditions appropriate for a first
20 amplification reaction, thereby producing a
set of first amplification products, with the
proviso that the conditions include a high
concentration of $MgCl_2$ and a low extension
temperature, whereby the target sequences in
25 the template nucleic acid are amplified;
 - (c) combining the first amplification products and
a plurality of pairs of labeled primers,
wherein each member of a labeled primer pair
30 comprises a constant segment, under conditions
appropriate for members of the labeled primer
pairs to hybridize to complementary nucleic
acid sequences on the first amplification
products sufficiently well to permit primer
extension by a polymerase enzyme, thereby
35 producing product-primer complexes; and

- (d) subjecting the product-primer complexes to conditions appropriate for a second amplification reaction, thereby producing a set of second amplification products which are labeled.
- 5
2. The method of claim 1, wherein the constant segment for the forward chimeric primer is 5'-TAATACGACTCACTATAGGGAGA-3' (SEQ ID NO:1) and the constant segment for the reverse chimeric primer is 10 5'-AATTAACCCTCACTAAAGGGAGA-3' (SEQ ID NO:2).
3. The method of claim 1, wherein the labeled primer pairs are labeled with a biotin molecule, a fluorophore, a dye, a metal, or a radionuclide.
4. The method of claim 1, wherein the $MgCl_2$ 15 concentration is from about 2.5 millimolar to about 7.0 millimolar.
5. The method of claim 4, wherein the $MgCl_2$ concentration is about 5 millimolar.
6. The method of claim 1, wherein the extension 20 temperature is from about 60°C to about 70°C.
7. The method of claim 6, wherein the extension temperature is from about 60°C to about 65°C.
8. The method of claim 7, wherein the extension temperature is about 65°C.
- 25 9. The method of claim 1, wherein the template nucleic acid is isolated nucleic acid.
10. The method of claim 9, wherein the template nucleic acid is isolated genomic DNA.

11. The method of claim 1, wherein the template nucleic acid is cDNA.
12. The method of claim 1, wherein the template nucleic acid is not isolated away from other cellular components.
13. A method for simultaneously amplifying a plurality of target sequences from template nucleic acid and labeling the amplification products, the method comprising:
- (a) combining template nucleic acid and a plurality of pairs of labeled chimeric primers, wherein each member of a labeled chimeric primer pair comprises a hybridization segment and a labeled constant segment, under conditions appropriate for members of the labeled chimeric primer pairs to hybridize to complementary nucleic acid sequences on the template nucleic acid sufficiently well to permit primer extension by a polymerase enzyme, thereby producing template-primer complexes; and
- (b) subjecting the template-primer complexes to conditions appropriate for an amplification reaction, thereby producing a set of labeled amplification products.
14. The method of claim 13, wherein the constant segment for the forward chimeric primer is 5'-TAATACGACTCACTATAGGGAGA-3' (SEQ ID NO:1) and the constant segment for the reverse chimeric primer is 5'-AATTAACCCTCACTAAAGGGAGA-3' (SEQ ID NO:2).

15. The method of claim 13, wherein the labeled primer pairs are labeled with a biotin molecule, a fluorophore, a dye, a metal, or a radionuclide.
- 5 16. The method of claim 13, wherein the amplification conditions include a high concentration of MgCl_2 .
17. The method of claim 16, wherein the MgCl_2 concentration is from about 2.5 millimolar to about 7.0 millimolar.
- 10 18. The method of claim 17, wherein the MgCl_2 concentration is about 5 millimolar.
19. The method of claim 13, wherein the first amplification conditions include a low extension temperature.
- 15 20. The method of claim 19, wherein the extension temperature is from about 60°C to about 70°C .
21. The method of claim 20, wherein the extension temperature is from about 60°C to about 65°C .
22. The method of claim 21, wherein the extension temperature is about 65°C .
- 20 23. The method of claim 13, wherein the template nucleic acid is isolated nucleic acid.
24. The method of claim 23, wherein the template nucleic acid is isolated genomic DNA.
- 25 25. The method of claim 13, wherein the template nucleic acid is cDNA.

26. The method of claim 13, wherein the template nucleic acid is not isolated away from other cellular components.
27. A kit for simultaneously amplifying a plurality of target sequences from a template nucleic acid and labeling the amplification products, the kit comprising
- (a) a plurality of pairs of chimeric primers, wherein each member of a chimeric primer pair comprises a hybridization segment and a constant segment, so that under appropriate conditions the members of the chimeric primer pairs can hybridize to complementary nucleic acid sequences on a template nucleic acid sufficiently well to permit primer extension by a polymerase enzyme; and
- (b) at least one reaction mixture appropriate for use in amplification reactions, with the proviso that at least one of the reaction mixtures includes a high concentration of $MgCl_2$;
- so that when template nucleic acid is combined with the chimeric primers of (a) and a reaction mixture of (b) under the appropriate conditions, a plurality of target sequences are amplified from the template nucleic acid.
28. The kit of claim 27, wherein the constant segment for the forward chimeric primer is 5'-TAATACGACTCACTATAGGGAGA-3' (SEQ ID NO:1) and the constant segment for the reverse chimeric primer is 5'-AATTAACCCTCACTAAAGGGAGA-3' (SEQ ID NO:2).
29. The kit of claim 27, wherein the constant segment is labeled.

30. The kit of claim 29, wherein the label is a biotin molecule, a fluorophore, a dye, a metal, or a radionuclide.
- 5 31. The kit of claim 27, wherein a reaction mixture with a high MgCl_2 concentration has an MgCl_2 concentration of about 2.5 to about 7.0 millimolar.
32. The kit of claim 31, wherein a reaction mixture with a high MgCl_2 concentration has an MgCl_2 concentration of about 5 millimolar.

1/4

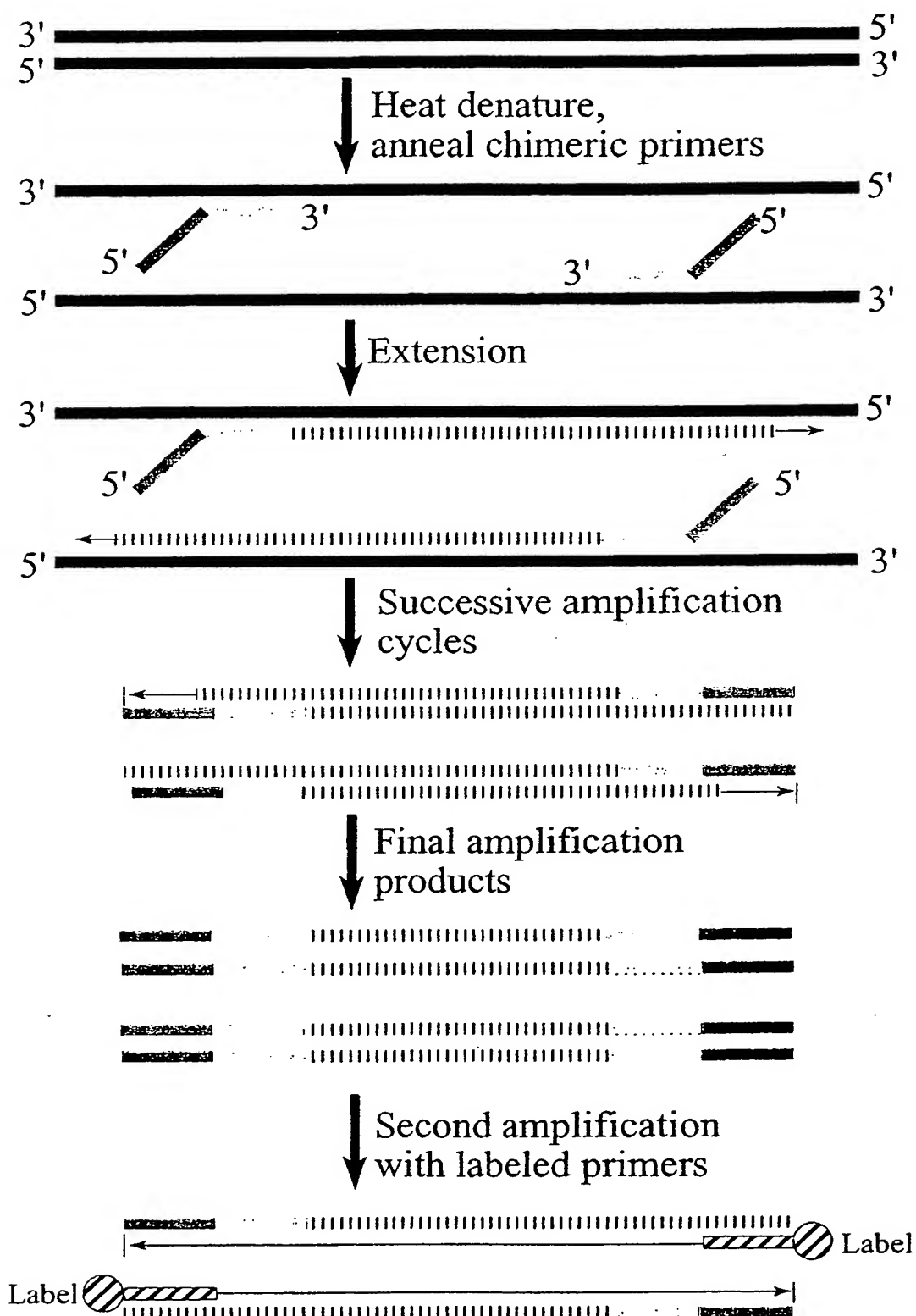
Fig. 1

Fig. 2A

FIRST AMPLIFICATION REACTION:

Original Template:

(SEQ ID NO: 6) <-- 3'-zzzz-ATTA-(target)-GCCG-zzzz-5' -->
 (SEQ ID NO: 7) <-- 5'-zzzz-TAAT-(target)-CGGC-zzzz-3' -->

↓
 Denature template,
 anneal chimeric primers

Initial Amplification Cycle:

(SEQ ID NO: 6) <-- 3'-zzzz-ATTA-(target)-GCCG-zzzz-5' -->
 5'-GGG-TAAT-3' →
 (SEQ ID NO: 7) <-- 5'-zzzz-TAAT-(target)-CGGC-zzzz-3' -->
 ← 3'-GCCG-TTT-5'

↓
 Products after first
 amplification cycle

(SEQ ID NO: 8) <-- 3'-zzzz-ATTA-(target)-CGGC-TTT-5'
 (SEQ ID NO: 9) 5'-GGG-TAAT-(target)-CGGC-zzzz-3' -->

↓
 Denature template,
 anneal chimeric primers

Second Amplification Cycle:

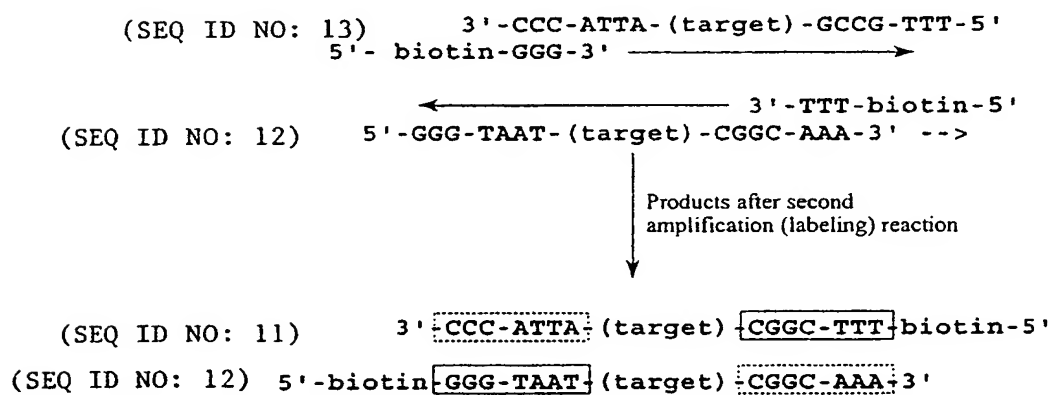
(SEQ ID NO: 10) <-- 3'-zzzz-ATTA-(target)-GCCG-TTT-5'
 5'-GGG-TAAT-3' →
 (SEQ ID NO: 9) 5'-GGG-TAAT-(target)-CGGC-zzzz-3' -->
 ← 3'-GCCG-TTT-5'

↓
 Products after successive
 amplification cycles

(SEQ ID NO: 11) 3'-CCC-ATTA-(target)-CGGC-TTT-5'
 (SEQ ID NO: 12) 5'-GGG-TAAT-(target)-CGGC-AAA-3'

Fig. 2B

SECOND AMPLIFICATION REACTION:



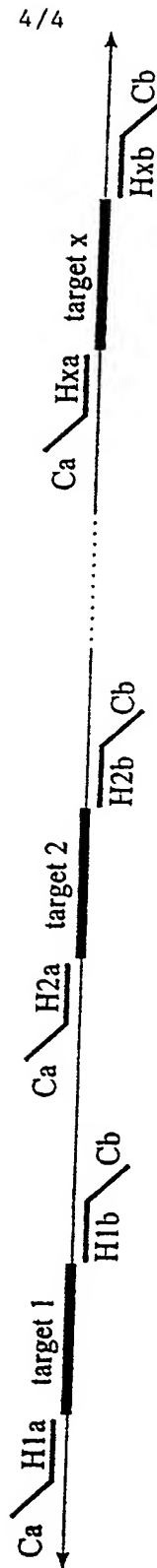


Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/10417A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C1201/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C120

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 41012 A (GENZYME CORP ; SHUBER ANTHONY P (US)) 19 December 1996 (1996-12-19) the whole document	1-32
X	SHUBER A P ET AL: "A SIMPLIFIED PROCEDURE FOR DEVELOPING MULTIPLEX PCRs" PCR METHODS & APPLICATIONS, vol. 5, no. 5, 1 December 1995 (1995-12-01), pages 488-493, XP000546034 ISSN: 1054-9803 the whole document	1-32

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "S" document member of the same patent family

Date of the actual completion of the international search

8 October 1999

Date of mailing of the international search report

15/10/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Hagenmaier, S

INTERNATIONAL SEARCH REPORT

International Application No

PC./US 99/10417

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RYCHLIK: "PRIMING EFFICIENCY IN PCR" BIOTECHNIQUES, vol. 18, no. 1, 1 January 1995 (1995-01-01), page 84, 86, 88/89 XP000486841 ISSN: 0736-6205 the whole document	
A	WO 95 11995 A (AFFYMAX TECH NV ;FODOR STEPHEN P A (US); GINGERAS THOMAS R (US); L) 4 May 1995 (1995-05-04) See p53 exon 7 T3 amplification primer, page 97.	
A	WO 91 18115 A (LIFE TECHNOLOGIES INC) 28 November 1991 (1991-11-28) See proto-promoter, example 7, page 29-30.	
A	US 5 624 825 A (NADEAU JAMES G ET AL) 29 April 1997 (1997-04-29) the whole document	
A	US 5 470 723 A (JURGENSEN STEWART R ET AL) 28 November 1995 (1995-11-28) the whole document	
P,X	WANG D G ET AL: "Large-scale identification, mapping, and genotyping of single -nucleotide polymorphisms in the human genome" SCIENCE, vol. 280, 15 May 1998 (1998-05-15), pages 1077-1082, XP002089398 ISSN: 0036-8075 the whole document	1-32

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/US 99/10417

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9641012 A	19-12-1996	US 5882856 A	16-03-1999
		AU 703417 B	25-03-1999
		AU 6163696 A	30-12-1996
		CA 2223729 A	19-12-1996
		EP 0832290 A	01-04-1998
		JP 11507226 T	29-06-1999
WO 9511995 A	04-05-1995	AU 8126694 A	22-05-1995
		EP 0730663 A	11-09-1996
		JP 9507121 T	22-07-1997
		US 5861242 A	19-01-1999
		US 5837832 A	17-11-1998
WO 9118115 A	28-11-1991	US 5194370 A	16-03-1993
		CA 2062963 A	17-11-1991
		EP 0483345 A	06-05-1992
		JP 5500012 T	14-01-1993
US 5624825 A	29-04-1997	US 5422252 A	06-06-1995
		AU 673424 B	07-11-1996
		BR 9402174 A	07-03-1995
		DE 69412540 D	24-09-1998
		DE 69412540 T	24-12-1998
		EP 0628640 A	14-12-1994
		ES 2122083 T	16-12-1998
		JP 2703183 B	26-01-1998
		JP 8019394 A	23-01-1996
		MX 9404063 A	31-01-1995
		SG 52767 A	28-09-1998
		US 5470723 A	28-11-1995
		US 5561044 A	01-10-1996
		US 5736365 A	07-04-1998
US 5470723 A	28-11-1995	US 5422252 A	06-06-1995
		US 5457027 A	10-10-1995
		AU 686169 B	05-02-1998
		AU 6888894 A	09-03-1995
		BR 9403324 A	11-04-1995
		CA 2129690 A	25-02-1995
		EP 0640691 A	01-03-1995
		JP 2814422 B	22-10-1998
		JP 7163396 A	27-06-1995
		SG 44897 A	19-12-1997
		US 5561044 A	01-10-1996
		US 5736365 A	07-04-1998
		AU 673424 B	07-11-1996
		BR 9402174 A	07-03-1995
		DE 69412540 D	24-09-1998
		DE 69412540 T	24-12-1998
		EP 0628640 A	14-12-1994
		ES 2122083 T	16-12-1998
		JP 2703183 B	26-01-1998
		JP 8019394 A	23-01-1996
		MX 9404063 A	31-01-1995
		SG 52767 A	28-09-1998
		US 5624825 A	29-04-1997
		AU 675503 B	06-02-1997
		AU 6058094 A	10-11-1994

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/10417

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5470723 A		CA 2121658 A,C	06-11-1994
		EP 0623682 A	09-11-1994
		JP 6343497 A	20-12-1994
		SG 50707 A	20-07-1998
		US 5840487 A	24-11-1998
		CA 2122203 A	12-11-1994
		EP 0624643 A	17-11-1994
		JP 2527533 B	28-08-1996
		JP 6319599 A	22-11-1994
		SG 44809 A	19-12-1997
		US 5536649 A	16-07-1996

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)